

Dynamic Precision Phenotyping Reveals Mechanism of Crop Tolerance to Root Herbivory

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Author Contributions

W.Q, M.P, T.G, B.R., L.K, A.S.C., A.T.K. Y.X., S.J.L. and J.S.F. contributed to developing the complex radiolabeled tracers used in this work. C.A.M.R., M.E. conducted plant tracer experiments and gene expression measurements. B.E.H provided the rootworms and treatment protocols. M.J.S. ran the cyclotron that produced the carbon-11 isotope. C.S. provided data management expertise for radioactive counting measurements. D.A. assisted in PET imaging. R.A.F supervised the research and wrote the manuscript with editing help from J.S.F., C.A.M.R. and M.E.

Short Title: Mechanisms of tolerance to root herbivory revealed

One-Sentence Summary: Whole-plant glutamine mobilization aids local-tissue, pathway-specific auxin biosynthesis in maize roots as a way to stimulate root re-growth after herbivory by the western corn rootworm.

ABSTRACT

The western corn rootworm (WCR) is a major pest of maize that is well adapted to most crop management strategies. Breeding for tolerance is a promising alternative to combat WCR, but is currently constrained by a lack of physiological understanding and phenotyping tools. We developed dynamic precision phenotyping approaches using carbon-11 with positron emission tomography, root autoradiography and radiometabolite flux analysis to understand maize tolerance to WCR. Our results reveal that WCR attack induces specific patterns of lateral root growth which are associated with a shift in auxin biosynthesis from indole-3-pyruvic acid to indole-3-acetonitrile. WCR attack also increases transport of newly synthesized amino acids to the roots, including the accumulation of glutamine. Finally, the regrowth zones of WCR attacked roots show an increase in glutamine turnover which strongly correlates with the induction of indole-3-acetonitrile-dependent auxin biosynthesis. In summary, our findings identify local changes in the auxin biosynthesis flux network as a promising marker for induced WCR tolerance.

Keywords: *Diabrotica virgifera virgifera*, western corn rootworm, crop tolerance, dynamic precision phenotyping, carbon-11, auxin biosynthesis flux network, glutamine mobilization

The western corn rootworm (Supplemental Fig. 1), *Diabrotica virgifera virgifera* LeConte, is a voracious pest of maize, *Zea mays* L.. Larvae hatch in the soil during late spring and

immediately begin feeding on the crop's root system. Over time, active feeding can result in substantial root damage with significant loss of water and/or nutrient uptake, thus weakening plants (Flint-Garcia *et al.*, 2009). Plants also become highly susceptible to lodging when major damage is inflicted upon the anchoring root system. Taken together, these effects can result in significant corn yield losses and management costs totaling between \$650 million to \$1 billion in the U.S. annually (Flint-Garcia *et al.*, 2009; Gray *et al.*, 2009).

History reveals the enormous resilience and adaptability of this pest and just how quickly it can evolve to overcome management strategies. For example, resistance to application of chemical pesticides, including cyclodienes (benzene hexachloride, aldrin) and organophosphates (methyl parathion), was seen over just a 10 year period of their use in Nebraska's corn fields during the 1950's and 1990's, respectively (Ball *et al.*, 1963; Meinke *et al.*, 1998). Alternate management practices including rotation of corn with other crops on a seasonal basis was generally considered the best choice for management since 1909 (Levine *et al.*, 2002). In east/central Illinois, 95-98% crop land had adopted a management strategy using only soybean as the rotation crop. Unfortunately, the enthusiastic adoption of this strategy over a broad area combined with the efficacy of the technique, created a strong selection that favored a less common *D. v. virgifera* phenotype with reduced egg laying fidelity to cornfields. Over time, natural selection afforded a strong reproductive advantage to females laying their eggs in soybean fields. Since the late 1990's, a strain of the western corn rootworm with resistance to crop rotation can be found in parts of Illinois, Indiana, and parts of bordering states (Gray *et al.*, 2009; Levine *et al.*, 2002).

More recently, *D. v. virgifera* resistance to deployed GMOs has been reported. First introduced into the market to target this pest back in 2003, genetically altered *Bt*-maize expressing one or more proteins from the soil bacteria, *Bacillus thuringiensis*, provided enhanced plant defenses to larval feeding. When a vulnerable insect ate the *Bt*-containing plant, the protein

became activated in its gut forming a toxin that paralyzed the digestive system and caused it to stop feeding. Unfortunately, resistance began to show within three generations of selection (Meihls *et al.*, 2008).

An alternative strategy to reduce the negative impact of *D. v. virgifera* attack without triggering counter adaptations in the pest is plant tolerance, which relies on a plant's capacity to maintain growth and yield even in the presence of substantial damage. While *D. v. virgifera* tolerant maize germplasms exhibiting slight to moderate tolerances to *D. v. virgifera* have been reported¹ more effective lines are needed. Unfortunately, we know very little about the underlying mechanisms for crop tolerance. Over the years, one resounding message has been that the physiological processes affected by herbivory should be better characterized before breeding tools can be leveraged in a rational way to generate improved varieties that maintain high yields under herbivore pressure (Riedell, 1990). Rational decision-making in the breeding selection process requires rigorous phenotyping: however, present phenotyping tools tell us little about the plasticity of root systems, especially when it comes to understanding mechanisms for crop tolerance to attack belowground. It was recently suggested that the timing for allocation of newly fixed carbon resources, as soluble sugars, between leaves, stalks and root systems, and their coordination with mobilization of other resources including amino acids may play significant roles in determining the ability of maize plants to survive an attack by *D. v. virgifera* (Orians *et al.*, 2011; Robert *et al.*, 2014).

In the present work, our systematic evaluation of the physiological, metabolic and genetic basis for root regrowth as a tolerance trait sheds new light on the regulation of the growth hormone, auxin (indole-3-acetic acid, IAA), and its role in this process. Radioactive decay of carbon-11 (β^+ emitter, $t_{1/2} = 20.4$ min), dynamic whole-plant positron emission tomography, root autoradiography, and radiometabolite flux analyses allowed us to map the transport, allocation

and metabolism of carbon and nitrogen resources against genetic and radiolabeled biochemical markers including, [^{11}C]indole-3-acetic acid, [^{11}C]indole, [^{11}C]indole-3-acetonitrile, [^{11}C]indole-3-acetamide and L-[5- ^{11}C]glutamine (Supplemental Fig. 2). Taken together, these tools enabled us for the first time to rigorously map out the auxin biosynthesis flux network at regional tissue levels, and in turn provide new insights on auxin regulation and its coordination with the availability of a key amino acid, L-glutamine. The developed phenotyping tools can now be employed for the rapid identification and selection of *D. v. virgifera* tolerant maize germplasm.

RESULTS

Root herbivore attack induces asymmetric formation of lateral root primordia

In the field, *D. v. virgifera* tolerant maize plants often display a pronounced increase in lateral root growth (Fig. 1a). Initial imaging studies set out to determine whether this trait can be characterized in a laboratory setting using 3-week old maize seedlings at their V2 stage. Seedlings at this stage of development have mature nodal or crown roots showing developing lateral root primordia (LRP) and lateral root structures (Fig. 1b). The high degree of LRP spatial symmetry (Fig. 1b) found in healthy nodal roots is lost upon larva feeding (Fig. 1c). After leaf administration of $^{11}\text{CO}_2$, autoradiographic imaging of the gross [^{11}C]photosynthate distribution reveals that in healthy undamaged roots, the lateral root meristems and LRP have a high accumulation of ^{11}C radioactivity indicating that these sites are strong sinks for resources (Fig. 1d). Furthermore, the spatial patterning of radioactivity in the healthy root image was highly symmetrical. In contrast to this, roots mildly damaged by herbivore feeding show a nonsymmetrical patterning of [^{11}C]photosynthate in the LRP (Fig. 1e). These LRP sites of high sink strength (and presumably high metabolic activity) coincide with high specific binding of [^{11}C]IAA both in healthy roots (Fig. 1f) and in mildly damaged roots (Fig. 1g) suggesting a

strong correlation between auxin signaling, cellular metabolism and compensatory growth as a form of herbivore tolerance.

Compensatory growth is associated with distinct shifts in auxin biosynthesis and transport

Root growth is in part determined by the hormone auxin. We therefore conducted a series of experiments to understand whether the observed regrowth phenotype is accompanied by changes in auxin biosynthesis and its distribution. Endogenous auxin (IAA) concentrations in nodal roots were seen to increase significantly (by 45%) as a function of root herbivore attack reaching levels of 0.051 ± 0.012 ng mgFW⁻¹ (Supplemental Fig. 3). Even so, this result says little about auxin biosynthesis and its regulation. IAA can be traced back to chorismic acid, which is biosynthesized *via* the shikimate pathway within the cell chloroplast (Fig. 2a). Chorismic acid undergoes rapid amination to anthranilic acid *via* the action of L-glutamine. Anthranilic acid is then transformed through several steps into different indole compounds and eventually into key aromatic amino acids including L-tryptophan (Trp), phenylalanine and tyrosine that are essential for primary and secondary metabolism in plants (Maeda and Dudareva, 2012).

Two major pathways for IAA biosynthesis have been postulated in higher plants (Fig. 2a) (McSteen, 2010; Normanly, 2010; Mashiguchia *et al.*, 2011; Won *et al.*, 2011; Ljung, 2013; Wang *et al.*, 2015): a Trp-dependent route including: (i) the indole-3-acetaldoxime (IAOx) pathway (ii) the indole-3-acetamide (IAM) pathway; (iii) the indole-3-pyruvic acid (IPyA) pathway; and (iv) the tryptamine (TAM) pathway as well as a Trp-independent route branching either from indole-3-glycerol phosphate or indole has also been suggested (Wright *et al.*, 1991). Because the scientific community has lacked a strong set biochemical markers enabling rigorous mapping of the IAA biosynthesis flux network, little is known about the importance of individual pathways as a function of environmental stress. We applied several [¹⁴C]indole-containing

tracers to rigorously map key branch points of this complex network (Reid *et al.*, 2011; Lee *et al.*, 2015a,b). Using [^{11}C]indole, we mapped the Trp-dependent IAA biosynthesis pathways occurring within the upper nodal root tissues 1 hour after incubation with tracer for two experimental conditions including; (i) unstressed (control) conditions (Fig. 2b), and (ii) biotic stress conditions elicited by WCR larvae feeding (Fig. 2c). We observed the following: (i) IAA biosynthesis in unstressed control roots is dominated by the IPyA pathway with no detectable contributions from the IAM pathway; (ii) IAA biosynthesis in herbivore stressed roots is dominated by the IAOx pathway (leading through IAN), and the IAM pathway is now active. These results prompted our further investigation into the kinetics of some of these pathways. Using [^{11}C]IAN, we mapped the temporal profile for the disappearance of this substrate in upper nodal roots with, and without WCR attack (Fig. 3a). The kinetic loss of substrate is 1st order, and the rate of metabolism increases significantly upon treatment. We also mapped the growth of the [^{11}C]IAA product derived from [^{11}C]IAN metabolism (Fig. 3b) and found that the rate of product formation increases significantly with treatment, but that the rate of free [^{11}C]IAA utilization (either through conjugation, metabolism or protein binding) increases significantly, as well. Finally, using the [^{11}C]IAM tracer (Fig. 3c), we verified that the IAM pathway is not active in unstressed control roots, but becomes active with treatment exhibiting 1st order kinetics for substrate metabolism.

Next, we spatially mapped the amount of [^{11}C]IAA that was formed from [^{11}C]IAN after 1 hour incubation with tracer (Fig. 4a) across three regions of the nodal root length including the upper proximal portion where lateral roots are visibly growing (zone 1), the mid-root section extending down to the elongation zone (zone 2), and the root tip (zone 3). Overall, our findings show a significantly higher rate of metabolic turnover of [^{11}C]IAN in the upper proximal zone, than in lower zones, as well as a significant treatment effect in this zone.

Because enzymes catalyzing the conversion of IAOx to IAN are not known, we decided to target the nitrilase genes which have been characterized in maize and may play an important role in the conversion of IAN to IAA. Nitrilase (NIT1 and NIT2) gene expression patterns were quantified by reverse transcriptase real-time polymerase chain reactions and mapped across the same three root zones as described above (Fig. 4b). NIT1 gene expression does not show a statistical difference across the three root zones, nor does root herbivore treatment impose any effect on this gene's expression level. NIT2 also doesn't exhibit spatial patterning in gene expression across the root zones for the unstressed controls. However WCR attack significantly lowers NIT2 expression in zones 2 and 3 relative to controls while possibly slightly increasing expression in zone 1, though this was not considered statistically significant ($P=0.062$).

Finally, re-distribution of [^{11}C]IAA *via* physical transport was measured using dynamic PET imaging (Fig. 5a). Using sub-cortical microliter injections of tracer in the upper root zone 1, PET imaging enabled us to visualize the movement of [^{11}C]IAA over time, as well as enabled us to quantify its transport speed (Fig. 5b). By measuring the time-of-arrival of tracer across two distinct ROIs, an auxin transport speed of $17.0 \pm 5.7 \text{ mm hr}^{-1}$ is observed for the unstressed control state. WCR attack significantly lowers transport by a factor of 2.6. Taken together, these results show that WCR attack leads to a pronounced reorganization of auxin biosynthesis and transport.

Root herbivore attack increases amino acid transport to the roots

The regulation of key hormones like auxin that are important for controlling root growth requires a steady supply of essential carbon and nitrogen resources. To understand how WCR influences resource allocation, we used $^{11}\text{CO}_2$ administered to source leaves in combination with dynamic PET imaging to trace the physiological and biochemical fate of 'new carbon' (as ^{11}C) at the

whole-plant level. We observed that allocation of gross [^{11}C]photosynthates belowground decreases significantly with WCR attack from 38.87 ± 4.23 % of fixed $^{11}\text{CO}_2$ to 25.91 ± 6.64 % (Fig. 6a). By contrast, transport speeds of gross [^{11}C]photosynthates more than doubled with herbivore treatment from 1.67 ± 0.24 to 3.43 ± 0.71 mm min $^{-1}$ (Fig. 6b), suggesting that the turnover of photosynthates increases under WCR attack.

Absolute amounts of ^{11}C -soluble sugars were measured both in the load (source) leaf at 10 and 90 min. post-administration of $^{11}\text{CO}_2$, as well as in the roots at 90 minutes, for control plants and plants subjected to root herbivore treatment (Fig. 7a). Gross export of ^{11}C -labeled sugars was calculated from the difference between the load leaf levels of ^{11}C -labeled sugars measured at the 10 and 90 min. time points (standard errors propagated). Three general observations are worth noting on the effects of root herbivore treatment on whole-plant sugar status: (i) it increases the flux of new carbon into source leaf soluble sugar pools; (ii) it increases the export of these sugar resources from those leaves (though not statistically significant); and (iii) it decreases their transport belowground to sites of attack. Specifically, gross sugar export from the source load leaf increases from 10.64 ± 2.04 % to 13.85 ± 2.66 % (based on total $^{11}\text{CO}_2$ fixed) as a function of treatment, but roots receive less of these sugars showing a significant decrease from 7.49 ± 0.91 to 4.60 ± 0.32 %. The relative profile of the individual sugars is also revealing. Here, we captured profile information for both the load leaf and the roots at 90 min (Fig. 7b). At the source leaf supply-side, root herbivore treatment significantly increases the metabolic partitioning of new carbon into [^{11}C]maltose and [^{11}C]sucrose, relative to controls, while generating a slightly higher proportion of [^{11}C]sucrose in the roots.

Similarly, absolute amounts of ^{11}C -labeled amino acids were measured in the load (source) leaf at 10 and 90 minutes post-administration of $^{11}\text{CO}_2$, as well as in the roots at 90 minutes, for control plants and plants subjected root herbivore treatment (Fig. 8a). Like before, the gross

export of ^{11}C -labeled amino acids was calculated from the difference between the levels of ^{11}C -labeled amino acids measured at the 10 and 90 minute time points. Three general observations are worth noting on the effects of WCR attack on whole-plant amino acid status: (i) it significantly increases the flux of new carbon into source leaf amino acid pools; (ii) it significantly increases the export of these resources from those source leaves; and (iii) it significantly increases their transport belowground to sites of attack. Specifically, gross amino acid export from the source leaf increases from $1.41 \pm 56\%$ to $4.21 \pm 0.89\%$ as a function of treatment. Roots receive more of these amino acids where allocations significantly increase from 0.47 ± 0.09 to $1.74 \pm 0.28\%$. Furthermore, studies using the L-[5- ^{11}C]glutamine tracer, applied to an abraded source leaf tip, show that root herbivore treatment doubles the phloem transport speed of this substrate (Supplemental Fig. 4). Like before, we captured amino acid profile information for the load leaf (Fig. 8b) and the roots (Fig. 8c) at 90 minutes. On the source leaf supply-side, root herbivore treatment significantly increases the metabolic partitioning of new carbon into [^{11}C]glutamine, [^{11}C]glutamate and [^{11}C]asparagine relative to controls. Roots also show a significantly higher proportion of [^{11}C]glutamine with treatment, but lower proportions of [^{11}C]alanine and other [^{11}C]-amino acids. These results show that WCR attack triggers strong and opposing changes in carbohydrate and amino acid allocation to the roots, which reflects the reorganization of root metabolism and growth upon herbivore infestation.

Changes in glutamine and auxin metabolism are strongly correlated

Because glutamine is important to maintaining auxin homeostasis (Ljung, 2013) through its involvement in the shikimate pathway (Maeda and Dudareva, 2012) providing indole-like substrates in support of auxin biosynthesis, we further investigated the dynamics of its metabolism in roots. Using the L-[5- ^{11}C]glutamine tracer, we mapped its metabolism to [^{11}C]glutamate as a function of root tissue type (upper nodal root versus lower root tip) and root

herbivore treatment (Fig. 9a). Results show that glutamine metabolism is significantly faster in upper root tissues than in tips. Furthermore, WCR attack increases metabolic turnover of L-[5-¹¹C]glutamine, for both of these tissue types. Lastly, by mapping L-[5-¹¹C]glutamine metabolism in upper root tissues (Fig. 9b) against [¹¹C]IAA biosynthesis (derived from the [¹¹C]IAN precursor) we see a strong correlation between the two processes and their response to WCR attack, suggesting that glutamine accumulation and IAA signaling are tightly coupled in maize roots.

DISCUSSION

Influence of Biotic Stress on Auxin Regulation

In an environment where higher plants are continuously subjected to a broad array of biotic and abiotic stimuli, their adaptability to such conditions requires a high degree of plasticity across scales of the whole plant. Plasticity, best reflected by rapid changes in a plant's physiology and/or central metabolism, can give rise to significant changes in growth and morphology, as well as in changes secondary metabolism. Often, active defensive strategies of a plant against would-be 'attackers' involves changes to secondary metabolism producing toxins that can repel, deter or kill their attacker (Howe and Jander, 2008). Defense can also involve tolerance mechanisms that allow the plant to regrow or reproduce after significant damage and/or tissue loss (Strauss and Agrawal, 1999). Underlying mechanisms for triggering such responses are traced to long-range signaling involving specialized hormones that help coordinate allocation of resources to targeted distal tissues and organs, as well as the metabolic partitioning of these resources (Thorpe *et al.*, 2007).

Hormones like auxin are known classically for their roles in regulating plant development including axis formation and patterning during post-embryogenesis, vascular elongation, leaf

expansion, inflorescence, fruit development, tropism and apical dominance (Woodward and Bartel, 2005; Kazan and Manners, 2009). Most particularly, auxin can stimulate ‘reshaping’ of root system architecture by triggering branch root patterning and root hair formation (McSteen, 2010).

The supply of auxin (IAA) to targeted tissues and organs of the plant is thought to rely on a fine balance between its biosynthesis and its physical transport both in and out of cells, as well as across tissues. Through a combination of efflux carrier proteins, including the PIN family and ABCB family (Zažimalová *et al.*, 2010) and influx carrier proteins, including the AUX1/LAX family (Swarup and Péret, 2012), polar auxin transport can be established at a cellular level providing the plant with a mechanism for effectively transporting this polar substrate across longer distances. Often, gradients in auxin concentration are found in targeted tissues which are thought to provide developmental cues for the plant (Blilou *et al.*, 2005; Benková *et al.*, 2009). For example, auxin is typically found at its highest concentration at the root tip (Agtuca *et al.*, 2014).

As described earlier (Fig. 2a), several pathways can play a role in producing auxin. Of these, we were primarily interested in the IAOx and IAM pathways. IAN is a putative IAA precursor that is synthesized from IAOx. Nitrilases (NITs) are important in converting IAN-to-IAA. Two nitrilase genes, ZmNIT1 and ZmNIT2, have been identified which are encoded in the maize genome and are expressed in developing kernels and seedlings (Park *et al.*, 2003). Prior research using a *Zmnit2* knockout maize mutant showed a significantly lower root IAA concentration than WT and it exhibited a strong root phenotype suggesting that a pathway through IAN is important (Kriechbaumer *et al.*, 2007). Indeed, our metabolic flux assays show that this pathway is the second most dominate pathway, next to the IPyA pathway, contributing to auxin in unstressed maize roots, and is the most dominant pathway when roots come under herbivore attack.

Furthermore, the regional specificity exhibited by this pathway, as evidenced by higher NIT2 gene expression and faster [^{11}C]IAN kinetics in the upper root zone where lateral root growth is evident, directly links this biochemical response to regrowth and crop tolerance. We note that past studies that spatially mapped both the IAA biosynthesis rate (Ljung *et al.*, 2005) and the expression level of key genes linked with auxin biosynthesis (Birnbaum *et al.*, 2003) across similar root zones in *Arabidopsis thaliana* found higher rates of auxin biosynthesis and higher levels of nitrilase expression in the upper zone where lateral root growth occurs. Hence, the significant down-regulation that we see in NIT2 expression in the lower maize root zones where no lateral root growth occurs suggests that the IAOx pathway may be uniquely tied to a plant stress response mechanism that aligns with the development lateral roots.

The IAM pathway, though considered an important pathway in bacteria, manifests in only very minute amounts of IAM in certain plants (Novák *et al.*, 2012). The IAM hydrolase (AtAMI1) enzyme, responsible for converting IAM-to-IAA, has also been observed in some plant species (Pollman *et al.*, 2006; Nemoto *et al.*, 2009). Even so, little is known about the importance of the IAM pathway across the plant kingdom, or of its connectivity with other pathway intermediates such as IAN in making IAA (see Fig. 2a). Through two different tracer experiments, our results clearly show that the IAM pathway is not active in unstressed plants, but activates under biotic stress. Even so, this pathway only contributes a minor portion of substrate to the overall auxin biosynthesis flux network. Furthermore, the 1st order kinetics for metabolism of both [^{11}C]IAN and [^{11}C]IAM substrates clearly indicates that pathway interconnectivity does not exist for this system. As noted in Figure 2, there has been some speculation on the interconnectivity of these pathways based on identification of key enzymes. However, we note that these precursors can also play important roles in plant secondary defense chemistry leading to defense compounds such as indole glucosinolates and camalexin, and

perhaps the existence of these enzymes in plants is to serve this purpose and not auxin biosynthesis (Ljung, 2013).

Influence of Biotic Stress on Central Metabolism and Resource Allocation

While resistance mechanisms have been extensively studied over the years, much less is known about the mechanistic basis of tolerance (Stowe *et al.*, 2000). Resource reallocation following real or simulated leaf attack aboveground has been well documented across numerous plant species, including tomato, tobacco, maize, barley, and poplar where the response has been increased export of leaf photosynthates to the stem and roots (Babst *et al.*, 2005; Babst *et al.*, 2008; Gómez *et al.*, 2010; Hanik *et al.*, 2010a,b; Henkes *et al.*, 2008; Schwachtje *et al.*, 2006). Similarly, nitrogen reallocation from leaves-to-roots was found to increase in tomato plants that were subjected to simulated herbivory aboveground (Gómez *et al.*, 2010). More recently, our work using real root herbivore treatments showed decreased allocation of gross [^{11}C]photosynthates from source leaves to nodal roots in maize, as well as decreased root meristematic activity (Robert *et al.*, 2012; Robert *et al.*, 2014). However, we have shown that both biotic (Hanik *et al.*, 2010a) and abiotic stresses (Pankiewicz *et al.*, 2015) can drastically and rapidly alter new carbon fluxes into key metabolite pools that make up plant photosynthates. Most particularly, increased partitioning of new carbon into ^{11}C -labeled amino acid pools were noted which coincided with compensatory reduction of the ^{11}C -labeled sugar pools. In the present work, we also observe a 2.6-fold increase in new carbon partitioning into source leaf ^{11}C -labeled amino acid pools as a function of root herbivory.

Unlike before, there was no compensatory decrease in the ^{11}C -labeled sugar pool, but rather a slight increase following WCR attack. This may be due to the fact that $^{11}\text{CO}_2$ fixation (ie. input of new carbon) actually increases when roots come under attack (Robert *et al.*, 2014)– a feature

that was not evident in prior aboveground herbivore studies. Upon closer inspection of our data, we begin to see an interesting story unfold where an increase in metabolic partitioning of new carbon into source leaf amino acids (specifically into highly transportable amino acids like glutamine) enables the plant to quickly translocate more nitrogen resources to the roots when they come under attack. This statement is substantiated by key observations including: (i) increases in allocation of ^{11}C -labeled amino acids to roots; (ii) increases in relative amounts of ^{11}C glutamine in the root amino acid pool; (iii) and increases in the rate of transport of the L-[5- ^{11}C]glutamine tracer. We suspect this strong physiological response is being driven by the increase in ^{11}C glutamine metabolism we see in roots when they come under attack. In concert with this, there is significant inhibition in the flow of sugars to roots. Because, the profile of ^{11}C -labeled sugars in these sink tissues reflect higher levels of ^{11}C sucrose, we suspect that this strong physiological response is driven by decreased sink demand for sugars. Indeed, our prior studies revealed a significant reduction in root invertase enzyme activity with root herbivore treatment (Robert *et al.*, 2014).

CONCLUSIONS

In summary, this work has revealed several new insights about root regrowth in crop tolerance to root herbivore attack as it relates to auxin biosynthesis and its regulation including: (i) auxin biosynthesis is tightly regulated at the local root tissue level with minimal hormone translocation away from site of biosynthesis; (ii) auxin biosynthesis is highly pathway specific, with the IAOx pathway through IAN dominating under stress; and (iii) auxin biosynthesis is tightly coordinated with glutamine mobilization to roots, and with increased glutamine metabolism at sites of regrowth.

Given the redundant nature through which auxin is biosynthesized in higher plants, it is unlikely that up-regulating a specific pathway using genetic engineering will satisfy the local tissue demands for this hormone. Other pathways will likely just down-regulate enabling the auxin flux network to maintain *status quo* based on the supply of substrates needed to support the overall process. A more viable approach is to target increasing the supply of these essential substrates. Our observations suggest that improving upon whole plant glutamine utilization through increased glutamine biosynthesis and/or transport would be a good place to start for crop breeding programs or for engineering new GMOs.

MATERIALS AND METHODS

Materials

All chemicals used in these studies were obtained from Sigma Aldrich (St. Louis, MO, USA) and were used without any further purification.

Plant Growth and Treatment

Corn kernels of the B73 line (USDA Agricultural Resource Services-Germplasm Resource Network, Ames, IA, USA) were germinated in darkness for 48 hr on wetted KimWipe™ tissues placed in 7 × 7 cm Petri-dishes. The dishes were wrapped with aluminum foil and kept at room temperature. After 48 hr, the Petri dishes were unwrapped and placed under Agro lights giving 100 μmol m⁻² s⁻¹ of light intensity during a 16 hr photoperiod. Once primary roots of germinating seedlings reached 1-2 cm in length they were transplanted into 600 mL glass growth cells filled with Hoagland's fortified agar gel.

Agar gels were prepared out of 3 L of de-ionized water, 4.9 g Hoagland modified basal salt mixture (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) and 1.66 g MES hydrate.

The pH of the solution was adjusted to 5.9 by adding 1N sodium hydroxide solution. While stirring, 8.4 g Gelzan CM (Sigma-Aldrich Corp. St. Louis, MO USA) were added. The solution was autoclaved (Harvey SterileMax, Thermo Fisher Scientific, Inc., Pittsburg, PA, USA) for 15 min at 121°C and mixed at high speed to enable aeration of the viscous solution before it had a chance to set as a gel. Sterilized plastic inserts were placed within each cell before the gel set. This allowed us to position the germinated kernel into a notch in the top portion of the cell. Following this, the tops of the cells were sealed with Parafilm™ and completely wrapped with aluminum foil to block out light. A small hole at the top of the cell enabled the developing plant to grow through. The cells were cultivated in growth chambers (Convion, Inc., Winnipeg, Manitoba, Canada) at 23°C using $320 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light intensity on a 12 hr photoperiod. Three-week old plants that measured ~ 35-40 cm in height were used for studies.

For root herbivore studies involving attack by *D. v. virgifera*, three-week old plants were subjected to feeding by twelve *D. v. virgifera* larvae at their 2nd instar stage. Larvae were allowed to free-feed for 4 days prior to screening plant physiological and biochemical responses using carbon-11.

¹¹CO₂ Production and Administration

¹¹CO₂ was produced *via* the ¹⁴N(p, α)¹¹C nuclear transformation from a 20 mL target filled with high-purity nitrogen gas containing 800 ppm oxygen (400 mL @ STP) using 18MeV protons from the TR-19 (Ebco Industries Ltd, Richmond, BC, Canada) cyclotron at BNL, and captured on a molecular sieve (4Å). The ¹¹CO₂ that was trapped on the molecular sieve was desorbed and quickly released into an air stream at 200 mL/min as a discrete pulse for labeling a leaf affixed within a 5 × 10 cm lighted ($320 \mu\text{mol m}^{-2} \text{s}^{-1}$) leaf cell at 21°C to ensure a steady level of fixation. The load leaf affixed within the cell was pulse-fed ¹¹CO₂ for 1 minute, then chased with normal

air for the duration of exposure. A PIN diode radiation detector (Carroll Ramsey Associates, Inc., Berkeley, CA, USA) affixed to the bottom of the leaf cell enabled continuous measurement of radioactivity levels within the cell during the initial pulse and in the minutes right after the pulse giving information on $^{11}\text{CO}_2$ fixation (Ferrieri *et al.*, 2005).

Radiosyntheses of Complex ^{11}C -Labeled Substrates

The radiosynthesis of indole-3- ^{11}C acetic acid (^{11}C -IAA) and its biosynthetic precursors, ^{11}C indole-3-acetonitrile (^{11}C -IAN) and indole-3- ^{11}C acetamide (^{11}C -IAM) using nucleophilic ^{11}C -cyanations on gramine was previously reported by us as a way to examine hormone signaling pathways in plants noninvasively (Reid *et al.*, 2011). In the past year, we further refined this original synthesis method to include a two-step, one-pot process with integrated SPEs that enabled reliable and remotely controllable production of these radiotracers in less than 1 hour yielding 0.22-0.46 GBq of product at specific activities of $47.4 \pm 12.5 \text{ GBq } \mu\text{mol}^{-1}$ and >98% radiochemical purity (Reid *et al.*, 2011; Lee *et al.*, 2015a). The improvements to radiochemistry allowed for final product formulation in extremely small volumes (0.15-0.3 mL of DI water at pH 6-7) that were suitable for *in vivo* PET plant imaging, and/or metabolic flux assays.

The radiosynthesis of [2- ^{11}C]indole was recently reported by us (Lee *et al.*, 2015b) which leveraged the nucleophilic ^{11}C -cyanation reaction on 2-nitrobenzyl bromide generating a highly reactive 2-(2-nitrophenyl)-[1- ^{11}C]acetonitrile intermediate which was subjected to nickel catalyzed reductive cyclization to yield the desired product in less than 1 hour at >98% radiochemical purity, and with a specific activity of $176 \pm 24.8 \text{ GBq } \mu\text{mol}^{-1}$.

A previously reported ^{11}C cyanide end-capping labeling method (Qu *et al.*, 2012) was recently refined by us (Gleede *et al.*, 2015) to create a milder, more reliable, two-step method

for radiosynthesizing 185-370 MBq batches of L-[5-¹¹C]-glutamine in less than 50 min at >93% radiochemical purity and possessing a L/D enantiomeric ratio of 94% ± 2.5 / 2.2% ± 1.7, and specific activity of 7.0 ± 1.5 GBq μmol⁻¹.

Complex organic radiotracers were introduced into targeted root tissues using sub-cortical injections (< 1 uL). We compared this method for tracer administration to that of grinding fresh tissue with tracer and obtained similar results on radiometabolite profiles.

Plant Imaging

We used MicroPET imaging (Concorde MicroSystems, Inc., Knoxville, TN, USA) to carry out 90 min dynamic scanning on roots. The system was used in its default configuration as described previously for all dynamic imaging experiments (Knoess *et al.*, 2003; Alexoff *et al.*, 2003). Images reconstructed by filtered back projection had a volumetric resolution of ~5 μL at the center of the field of view increasing to ~30 μL at a radius of 4 cm (Knoess *et al.*, 2003). Data corrections for random coincidences and dead-time losses were provided by the manufacturer. Scattered coincidences were subtracted using a tail fitting method described previously (Alexoff *et al.*, 2003). ASIPro VM software (RSI Research Systems, Inc., Boulder, CO, USA) was used to create regions of interest (ROI) on reconstructed images giving information on radioactivity time-of-arrival between those ROIs. Distances between ROIs were also measured in pixels using the same software and related to mm spatial scales for calculating velocities (mm min⁻¹). After the microPET imaging, plants were removed from their growth cells and selected tissues (roots and leaves) were imaged using autoradiography (Typhoon 7000: GE Healthcare, Piscataway, NJ, USA). Image data was quantified using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

[¹¹C]Sugar and [¹¹C]Amino Acid Analyses

Targeted tissues were extracted in 4× w/v of methanol, briefly vortexed (VWR analog vortex mixer; Sigma-Aldrich Corp. St. Louis, MO, USA) and then sonicated (Branson Bransonic 32; Sigma-Aldrich Corp. St. Louis, MO, USA) in an iced water bath for 10 min with intermittent vortexing to ensure complete mixing. The tubes were centrifuged (Eppendorf Centrifuge 5424) for 2 min at 15,000 rpm and the supernatant was filtered through 0.2 µm Acrodiscs (Gelman Sciences, Ann Arbor, MI, USA). The pellet contained all insoluble components which comprised mostly cell-wall polymers and starch. The filtrate contained small soluble compounds, including soluble sugars and amino acids. Sugars were separated and analyzed by thin layer chromatography (TLC) (Babst *et al.*, 2013). Glass backed NH₂-silica HPTLC-plates (200µm, w/UV254) were used for the sugar separation (Sorbent Technologies, Atlanta, GA, USA). Plates were pre-spotted with sugar standards of maltose, sucrose, fructose, glucose and xylose. Aliquots of radioactive tissue extract were applied to TLC plates using a semi-automatic Linomat 5 sample applicator (Camag Scientific, Inc., Wilmington, NC, USA) for high precision of spot size and sample volume. TLC plates were developed using a mobile phase consisting of 75:25 acetonitrile:water (v/v). Developed plates were imaged using autoradiography to determine the fraction of each radiolabeled sugar. The Plates were then heat-treated (200°C for 10 min) to initiate chemical reaction of individual sugars with the amino functionalized silica-support that gave fluorescence under long wavelength (365 nm) UV light providing co-registration of the ¹²C-sugar standards with individual ¹¹C-labelled components. ImageQuant TL software 7.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to analyze the radiographic image data to determine the amount of ¹¹C within each sugar.

An additional 150 µL of tissue extract was also delivered into 2 mL brown-glass vials (Fisher Scientific, Inc., Pittsburgh, PA, USA) where equal volumes of o-phthaldehyde amino acid derivatizing reagent (OPA) containing 0.1% (v/v) mercaptoethanol (Sigma Life Science, St.

Louis, MO, USA) were delivered. Mixtures were vortexed, and then allowed to react at ambient temperature for 3 min. 50 μ L of the derivatized samples were injected onto a reversed-phased analytical HPLC column (Phenomenex, Torrance, CA, USA: Ultramex™ C18, 10 μ m particle size, 250 mm \times 4.6 mm i.d.) using a pre-column gradient mixer (Sonntek, Inc., Upper Saddle River, NJ, USA) and a mobile phase comprised of A (DI water), B (0.01M NaH₂PO₄-buffered at pH 6.8 using trifluoroacetic acid) and C (methanol). At injection, the mobile phase (1.5 mL min⁻¹) was sustained at 75% A; 25% B for 5 min and then programmed to attain 20% B; 80% C by 30 min. Retention times of OPA-derivatized amino acid were measured against authentic standards using a fluorescence detector (280 nm excitation, 350 nm emission, Hitachi LaChrom Elite L-2485: purchased from Sonntek, Inc., Upper Saddle River, NJ, USA). The outlet of the fluorescence detector was connected in series to a NaI gamma radiation detector (Ortec Inc, Oak Ridge, TN, USA) that enabled direct measurement of the amount of radioactivity associated with each substrate eluting the column.

[¹¹C]IAA and Related [¹¹C]Indole Compound Analyses

Root tissues were subjected to the same methanol extraction procedure as described above. However, the alcoholic extracts were then subjected to a phase separation using equal volumes of ethyl acetate to remove undesirable sugars and amino acids from the subsequent analysis. Ethyl acetate fractions were reduced to dryness in a vacuum centrifuge (LabConco, Inc., Kansas City, MO, USA), and then reconstituted in 50 μ L methanol where aliquots measured on a gamma counter carbon-11 activity as well as injected onto a radio HPLC. The radio HPLC gradient system was identical to that described above, with the exception of the column and mobile phase used (Phenomenex, Torrance, CA, USA: Luna PFP (2), 5 μ m particle size, 250 \times 4.6 mm i.d.). The mobile phase was comprised of A (2.5% aqueous acetic acid adjusted to pH

3.8 using 1N KOH), B (80:20 acetonitrile:water). At injection, the mobile phase (1.0 mL min⁻¹) was programmed to adjust from 80% A; 20% B, to 50% A; 50% B over 25 minutes.

Two radio-TLC methods were also deployed to enable rapid assay of pathway-specific auxin biosynthesis kinetic fluxes using [¹¹C]IAN and [¹¹C]IAM radiotracers. In the first method, the silica TLC plate was developed using 1.5:1, hexane:ethyl acetate (0.1% formic acid v/v) as the solvent, which allowed rapid separation of [¹¹C]IAN and [¹¹C]IAA and leaving [¹¹C]IAM at the origin. In the second method, the silica TLC plates were pre-treated using NH_{3(aq)} (29%) and developed using pure ethyl acetate solvent to enable a different R_f elution profile of the three components with [¹¹C]IAN carried with the solvent front. Activity distributions on the plates were made visible by autoradiography and quantified using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Endogenous Auxin Hormone Analysis

Root tissues were placed in Eppendorf tubes and ground to a fine powder at liquid nitrogen temperature using a Retsch ball mill grinder (Verder Scientific, Inc., Newtown, PA, USA). 500 µL of 0.05M sodium phosphate buffer, pH 7.0, containing 0.02% (w/v) of sodium diethyldithiocarbamate as antioxidant and 250 ng of anthranilic acid (Sigma-Aldrich, Inc., St. Louis, MO, USA) as an internal standard were added to the Eppendorf tube, and the sample was extracted under continuous shaking for 1 hr at 4°C. After extraction, the pH was adjusted to approximately 2.6 with 1 M HCl, and the sample was slurried with 35 mg of Amberlite™ XAD-7HP (weakly polar polymeric resin, 20-60 mesh; Sigma-Aldrich, Inc., St. Louis, MO, USA) for 30 min. After removal of the buffer, the XAD-7 was washed twice using 500 µL of 1% acetic acid before being slurried twice more using 500 µL of dichloromethane for 30 min. The combined dichloromethane fractions were reduced to dryness in a vacuum centrifuge

(LabConco, Inc., Kansas City, MO, USA) and then trimethylsilylated with 25 μ L of acetonitrile and 25 μ L of *N,O*-Bis(trimethylsilyl)-trifluoroacetamide with 1 % (v/v) trimethylchlorosilane (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 70°C for 15 min. After evaporation to dryness (LabConco, Inc., Kansas City, MO, USA), samples were dissolved in hexane. 1 μ L aliquots were split injected (100:1 v/v) onto a Hewlett-Packard 5890A capillary gas chromatograph equipped with a 30 m HP-5 column (0.25 mm diameter, 0.25 μ m film thickness: Agilent Technologies, Inc., Santa Clara, CA, USA) and a nitrogen-phosphorous detector.

Quantitative RT-PCR Analysis

The nitrilase NIT1 and NIT2 gene expression patterns along three root zones (including upper proximal, mid-root and root tip) were quantified by performing a quantitative reverse transcriptase real-time polymerase chain reactions (Q-RT-PCR) as previously described (Erb *et al.*, 2010). The gene specific primers were used for NIT1 and NIT2 as previously described (Park *et al.*, 2003). The final Ct values were corrected for the housekeeping genes Actin and GapC and normalized to controls.

Statistical Analysis

Data was subjected to the Student t-test for unpaired samples assuming an unequal variance. Statistical significance levels were assigned to the following rating scale (*, $P < .05$; **, $P < .01$; ***, $P < .001$).

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FIGURE CAPTIONS

Figure 1. Panel a: ectopic branch root patterning of maize plant observed in the U. Missouri planting fields after infestation by *D. v. virgifera*. Panels b & c: photographs of healthy and damaged nodal roots. Loss of lateral root spatial symmetry is noted when roots were damaged by herbivore feeding. Panel d & e: root radiographic images showing [^{11}C]photosynthate distributions in healthy (Panel d) and damaged (Panel e) nodal roots. Images were acquired 90 minutes after administration of a dose of $^{11}\text{CO}_2$ to a source leaf. ^{11}C activity shows good spatial correlation with sites of lateral root primordia (LRP) suggesting these are sites are strong sinks with high metabolic activity. Panel f: root radiographic image acquired after a sub-cortical injection of [^{11}C]IAA into a healthy undamaged root – a similar symmetrical spatial patterning of ^{11}C tracer binding is seen between this image and the healthy undamaged root image presented in Panel d for [^{11}C]photosynthate. Panel g: root radiographic image acquired after a sub-cortical injection of [^{11}C]IAA into a WCR damaged root – a similar nonsymmetrical spatial patterning of ^{11}C tracer binding is seen between this image and the damaged root image presented in Panel e for [^{11}C]photosynthate. The spatial correlations of ^{11}C radioactivity seen between [^{11}C]IAA and [^{11}C]photosynthate images suggests that LRP are sites of high auxin receptor density.

Figure 2. Panel a: The biosynthesis of IAA precursors, such as indole-3-glycerol phosphate and L-Trp, takes place in plastids. L-Trp, the major IAA precursor, is generated via the shikimate pathway. The subsequent L-Trp-dependent IAA biosynthesis pathways are believed to occur in the cytosol. Four putative pathways for L-Trp-dependent IAA biosynthesis in higher plants are shown: the IAOx, IAM, IPyA and TAM pathways. The enzymes known to operate in each pathway are shown in italics. Solid pathway arrows reflect presumably active processes. Dashed pathway arrows are suggested to exist, but have yet to be proven. This panel reflects modifications to past published pathways (McSteen, 2010; Normanly, 2010; Ljung, 2013). Abbreviations: IAA, indole-3-acetic acid; IAAlD, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IPyA, indole-3-pyruvic acid; L-Trp, L-tryptophan; NITs, nitrilases; TDCs, tryptophan decarboxylases; TAM, tryptamine. Panel b & c: the IAA biosynthesis flux network across the four L-Trp-dependent pathways was quantified using [^{11}C]indole. Measurements were taken 1 hour after incubation with tracer. In unstressed control plants (panel b), the IPyA-pathway was dominant, the IAOx-pathway through IAN was second in importance, the TAM pathway was minor and the IAM pathway was not active. In herbivore stressed plants (panel c) the IAOx-pathway through IAN became the dominant pathway and the IAM pathway was now active.

Figure 3. Panel a: logarithmic plot of [^{11}C]IAN levels after subcortical injection of tracer into the upper proximal zone of a nodal root (N=5, control; N=7, root herbivory). Results reflect 1st order kinetics for [^{11}C]IAN metabolism and a strong root herbivore treatment effect with increased rate of substrate metabolism. Panel b: relative percent of [^{11}C]IAA product derived from [^{11}C]IAN metabolism shown in panel a. Herbivory increases the rate of [^{11}C]IAA formation as well as increases loss of free [^{11}C]IAA from metabolism, conjugation and/or

receptor binding. Panel c: logarithmic plot of [^{11}C]IAM levels after subcortical injection of tracer into the upper proximal zone of a nodal root (N=4, control; N=4, root herbivory). Results show that [^{11}C]IAM is not metabolized in unstressed control roots, but is actively metabolized in 1st order kinetics after root herbivore treatment.

Figure 4: Panel a. Spatial mapping of [^{11}C]IAA biosynthesis from [^{11}C]IAN across three nodal root zones including the upper proximal region (zone 1), the mid-root region including the elongation zone (zone 2) and the root tip (zone 3) (N=5, control; N=6, root herbivory). Higher levels of [^{11}C]IAN metabolism are indicated in zone 1. Root herbivore treatment significantly increased tracer metabolism in this zone, but had no effect in zones 2 & 3. Panel b: Ln-fold change in NIT1 and NIT2 gene expression measured across the same three root zones as a function of root herbivore treatment. No change in NIT1 gene expression was noted across any of the zones or as a function of treatment (N=3). On the other hand, NIT2 gene expression was significantly down-regulated with herbivore treatment in zones 2 & 3, but exhibited a slight up-regulation of expression in zone 1 (N=4), though not statistically significant (P=0.062). Where appropriate, statistical significance was denoted as: *, P \leq 0.05; **, P \leq 0.01; ***, P \leq 0.001.

Figure 5. Panel a: Projection Positron Emission Tomography (PET) image showing [^{11}C]IAA movement from the injection sites in the upper proximal zone of undamaged (control) nodal roots. Panel b: time-activity plots reflecting levels of decay corrected [^{11}C]IAA activity at the injection site over time in seconds (N=4, control; N=5, root herbivory). Root herbivory decreased [^{11}C]IAA transport 2.6-fold from an average speed of 17.0 \pm 5.7 mm hr⁻¹ as measured from time-of-arrival of the activity from across two distinct ROIs along an individual root.

Figure 6. Panel a: allocation belowground of gross [^{11}C]photosynthates derived from fixed $^{11}\text{CO}_2$. Root herbivore treatment significantly decreased belowground allocation of these resources. Panel b: the speed of [^{11}C]photosynthate transport (mm min^{-1}) shown in panel b increased significantly with treatment. Where appropriate, statistical significance was denoted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Figure 7. Panel a: levels of ^{11}C -labeled soluble sugars (presented as % total ^{11}C activity fixed as $^{11}\text{CO}_2$) were plotted for the following tissues: load leaf at 10 min. post-administration of $^{11}\text{CO}_2$; load leaf at 90 min. post-administration of $^{11}\text{CO}_2$; and roots at 90 min. post-administration of $^{11}\text{CO}_2$ to leaves. The gross export of ^{11}C -labeled soluble sugars was calculated from the difference in load leaf activities at 10 and 90 minutes. Root herbivore treatment manifested in a significant reduction in root allocation of ^{11}C -labeled soluble sugars. Panel b: relative distributions of ^{11}C -labeled soluble sugars are presented for the load leaf and roots at 90 min. post-administration of $^{11}\text{CO}_2$ and as a function of root herbivore treatment.

Figure 8. Panel a: levels of ^{11}C -labeled amino acids (presented as % total ^{11}C activity fixed as $^{11}\text{CO}_2$) were plotted for the following tissues: load leaf at 10 min. post-administration of $^{11}\text{CO}_2$; load leaf at 90 min post-administration of $^{11}\text{CO}_2$; and roots at 90 min. post-administration of $^{11}\text{CO}_2$ to leaves. The gross export of ^{11}C -labeled amino acids was calculated from the difference in load leaf activities at 10 and 90 minutes. Root herbivore treatment manifested in a significant increase in gross export of amino acids from source leaves, as well as in a significant increase in their allocation to roots. Relative distributions of ^{11}C -labeled amino acids are presented for the load leaf (Panel b) and roots (Panel c) at 90 min. post-administration of $^{11}\text{CO}_2$ and as a function

of root herbivore treatment. Treatment increased source leaf levels of [^{11}C]glutamine, [^{11}C]glutamate and [^{11}C]asparagine. Treated roots showed significantly higher levels of [^{11}C]glutamine, as well as lower levels of [^{11}C]alanine and other less abundant radiolabeled amino acids that were summed together. Where appropriate, statistical significance was denoted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Figure 9. Panel a: [^{11}C]Glutamine metabolism, represented as the ratio of [^{11}C]GLU-to-[^{11}C]GLN, was plotted against tracer incubation for upper root tissues and root tips as a function of root herbivore treatment. Two key observations include: (i) GLN metabolism is higher in upper root tissues than in tips; (ii) root herbivore treatment increases GLN metabolism across all spatial zones of the root. Panel b. [^{11}C]GLN metabolism, represented as the ratio of [^{11}C]GLU-to-[^{11}C]GLN was plotted against [^{11}C]IAN metabolism, represented as the ratio of [^{11}C]IAA-to-[^{11}C]IAN, as a function of root herbivore treatment. Regression analysis reveals a strong correlation between the two biochemical processes as well as a significant positive herbivore treatment effect. Abbreviations: GLN, glutamine; IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile.

SUPPLEMENTAL DATA

Supplemental Figure 1. Panel a: adult beetle of *Diabrotica virgifera virgifera*, LeConte. Panel b: immature larva of *D. v. virgifera*.

Supplemental Figure 2. Complex organic ^{11}C -labeled radiotracers used in this study.

Supplemental Figure 3. Endogeneous nodal root auxin (IAA) concentrations presented as ng mgFW^{-1} and plotted as a function of root herbivore treatment.

Supplemental Figure 4. Transport speed (mm min^{-1}) of L-[5- ^{11}C]glutamine plotted as a function of root herbivore treatment.

Supplemental Figure 1. Panel a: adult beetle of *Diabrotica virgifera virgifera*, LeConte. Panel b: immature larva of *D. v. virgifera*.

Supplemental Figure 2. Complex organic ^{11}C -labeled radiotracers used in this study. The carbon atom highlighted in red shows the sight where the radioisotope was inserted into the substrate skeleton.

Supplemental Figure 3. Endogeneous nodal root auxin (IAA) concentrations presented as ng mgFW^{-1} and plotted as a function of root herbivore treatment. Statistical significance was denoted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Supplemental Figure 4. Transport speed (mm min^{-1}) of L-[5- ^{11}C]glutamine plotted as a function of root herbivore treatment. Statistical significance was denoted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

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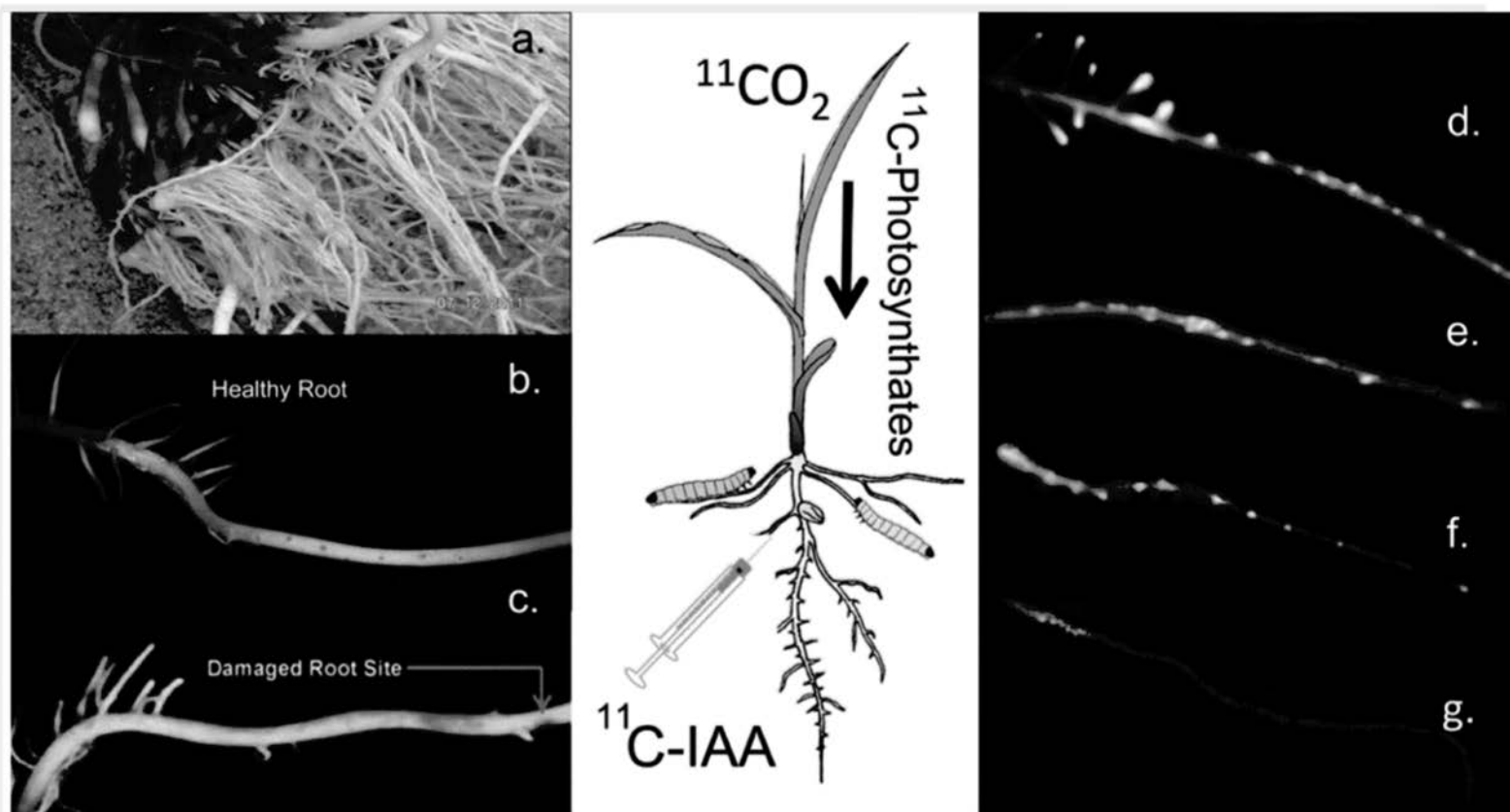


Figure 1. Panel a: ectopic branch root patterning of maize plant observed in the U. Missouri planting fields after infestation by *D. v. virgifera*. Panels b & c: photographs of healthy and damaged nodal roots. Loss of lateral root spatial symmetry is noted when roots were damaged by herbivore feeding. Panel d & e: root radiographic images showing [^{11}C]photosynthate distributions in healthy (Panel d) and damaged (Panel e) nodal roots. Images were acquired 90 minutes after administration of a dose of $^{11}\text{CO}_2$ to a source leaf. ^{11}C activity shows good spatial correlation with sites of lateral root primordia (LRP) suggesting these are sites are strong sinks with high metabolic activity. Panel f: root radiographic image acquired after a sub-cortical injection of [^{11}C]IAA into a healthy undamaged root – a similar symmetrical spatial patterning of ^{11}C tracer binding is seen between this image and the healthy undamaged root image presented in Panel d for [^{11}C]photosynthate. Panel g: root radiographic image acquired after a sub-cortical injection of [^{11}C]IAA into a WCR damaged root – a similar nonsymmetrical spatial patterning of ^{11}C tracer binding is seen between this image and the damaged root image presented in Panel e for [^{11}C]photosynthate. The spatial correlations of ^{11}C radioactivity seen between [^{11}C]IAA and [^{11}C]photosynthate images suggests that LRP are sites of high auxin receptor density.

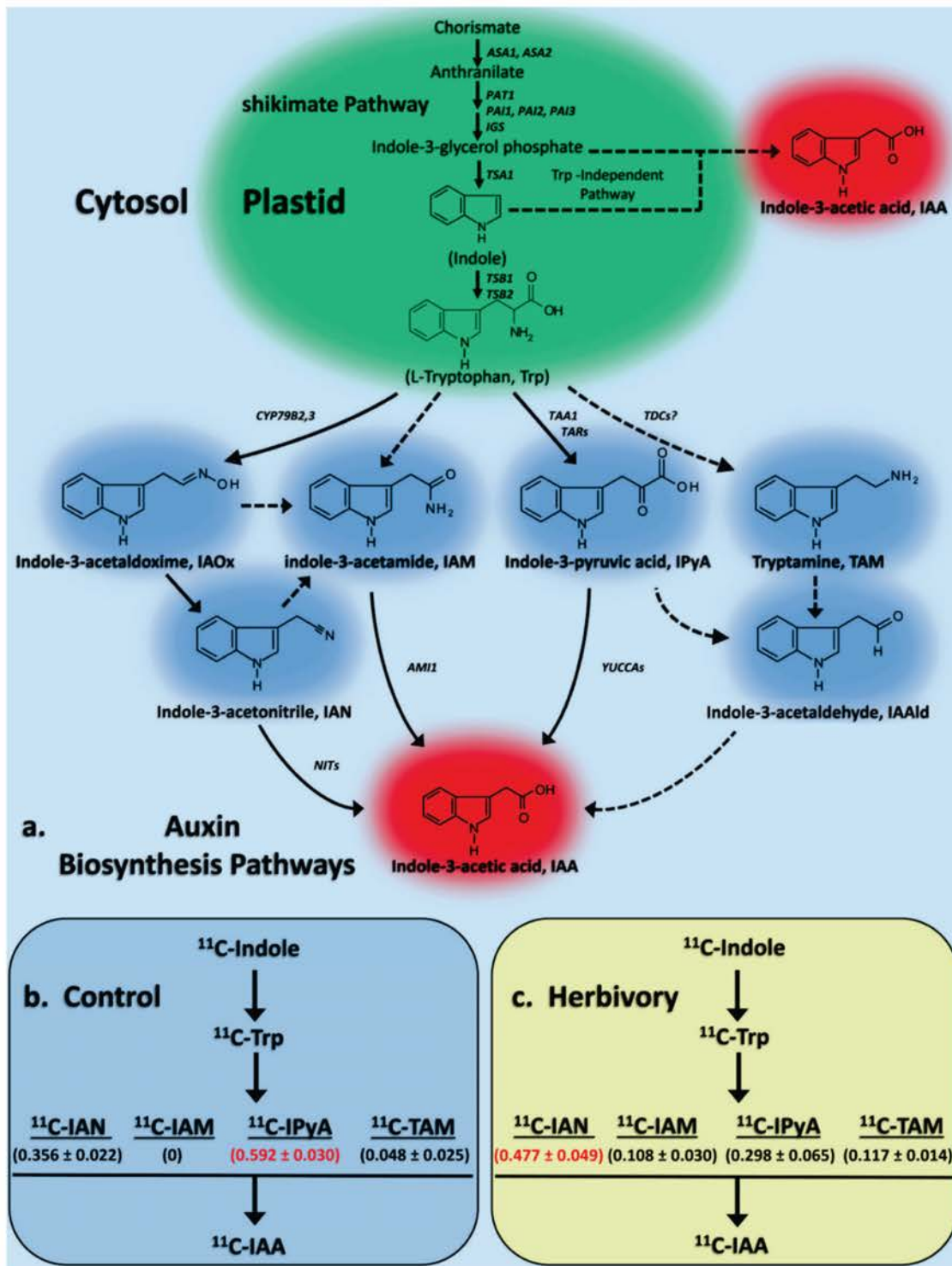


Figure 2. Panel a: The biosynthesis of IAA precursors, such as indole-3-glycerol phosphate and L-Trp, takes place in plastids. L-Trp, the major IAA precursor, is generated via the shikimate pathway. The subsequent L-Trp-dependent IAA biosynthesis pathways are believed to occur in the cytosol. Four putative pathways for L-Trp-dependent IAA biosynthesis in higher plants are shown: the IAOx, IAM, IPyA and TAM pathways. The enzymes known to operate in each pathway are shown in italics. Solid pathway arrows reflect presumably active processes. Dashed pathway arrows are suggested to exist, but have yet to be proven. This panel reflects modifications to past published pathways (McSteen, 2010; Normanly, 2010; Ljung, 2013). Abbreviations: IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IPyA, indole-3-pyruvic acid; L-Trp, L-tryptophan; NITs, nitrilases; TDCs, tryptophan decarboxylases; TAM, tryptamine. Panel b & c: the IAA biosynthesis flux network across the four L-Trp-dependent pathways was quantified using [^{11}C]indole. Measurements were taken 1 hour after incubation with tracer. In unstressed control plants (panel b), the IPyA-pathway was dominant, the IAOx-pathway through IAN was second in importance, the TAM-pathway was minor and the IAM pathway was not active. In herbivore-stressed plants (panel c) the IAOx-pathway through IAN became the dominant pathway and the IAM pathway was now active.

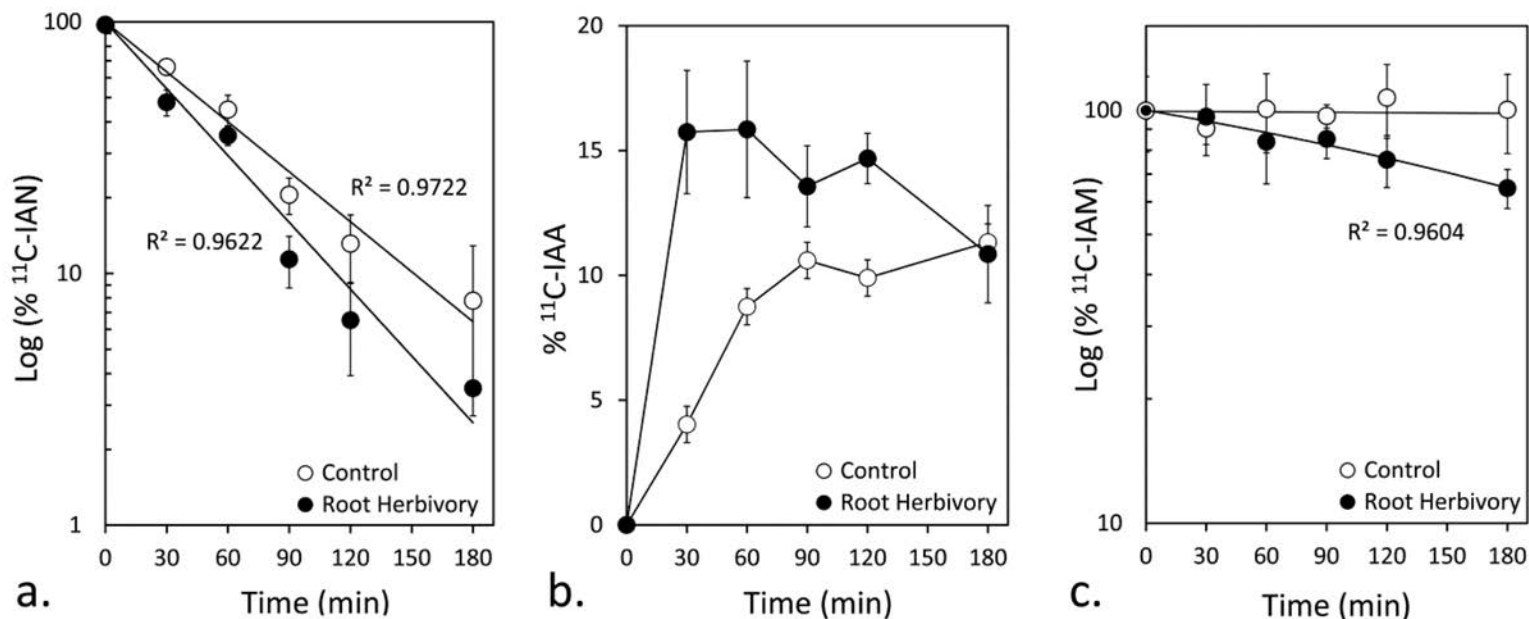


Figure 3. Panel a: logarithmic plot of $[\text{11C}]\text{IAN}$ levels after subcortical injection of tracer into the upper proximal zone of a nodal root (N=5, control; N=7, root herbivory). Results reflect 1st order kinetics for $[\text{11C}]\text{IAN}$ metabolism and a strong root herbivore treatment effect with increased rate of substrate metabolism. Panel b: relative percent of $[\text{11C}]\text{IAA}$ product derived from $[\text{11C}]\text{IAN}$ metabolism shown in panel a. Herbivory increases the rate of $[\text{11C}]\text{IAA}$ formation as well as increases loss of free $[\text{11C}]\text{IAA}$ from metabolism, conjugation and/or receptor binding. Panel c: logarithmic plot of $[\text{11C}]\text{IAM}$ levels after subcortical injection of tracer into the upper proximal zone of a nodal root (N=4, control; N=4, root herbivory). Results show that $[\text{11C}]\text{IAM}$ is not metabolized in unstressed control roots, but is actively metabolized in 1st order kinetics after root herbivore treatment.

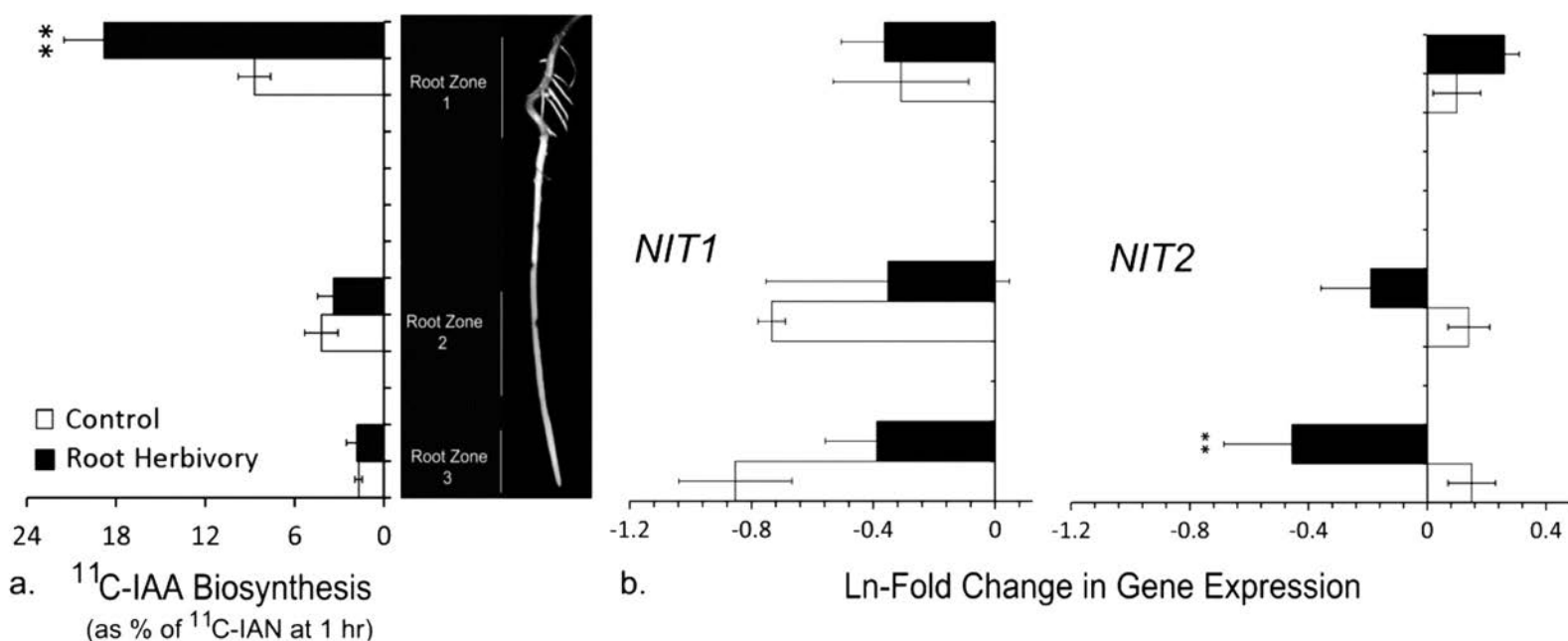
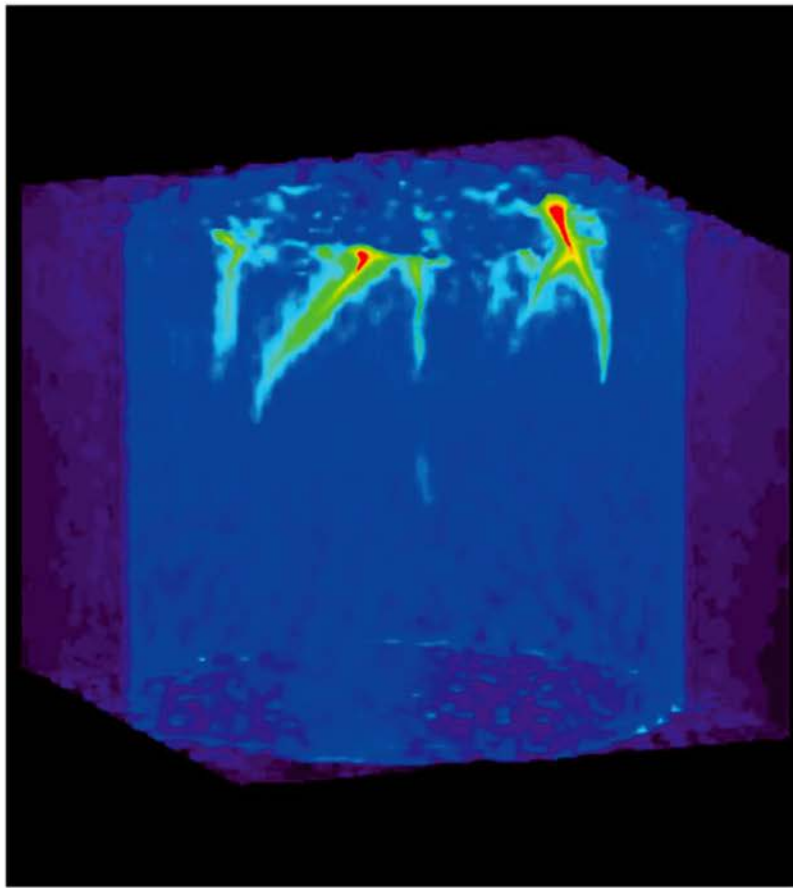


Figure 4: Panel a. Spatial mapping of [^{11}C]IAA biosynthesis from [^{11}C]IAN across three nodal root zones including the upper proximal region (zone 1), the mid-root region including the elongation zone (zone 2) and the root tip (zone 3) (N=5, control; N=6, root herbivory). Higher levels of [^{11}C]IAN metabolism are indicated in zone 1. Root herbivore treatment significantly increased tracer metabolism in this zone, but had no effect in zones 2 & 3. Panel b: Ln-fold change in NIT1 and NIT2 gene expression measured across the same three root zones as a function of root herbivore treatment. No change in NIT1 gene expression was noted across any of the zones or as a function of treatment (N=3). On the other hand, NIT2 gene expression was significantly down-regulated with herbivore treatment in zones 2 & 3, but exhibited a slight up-regulation of expression in zone 1 (N=4), though not statistically significant (P=0.062). Where appropriate, statistical significance was denoted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



PET Image of ^{11}C -IAA Transport

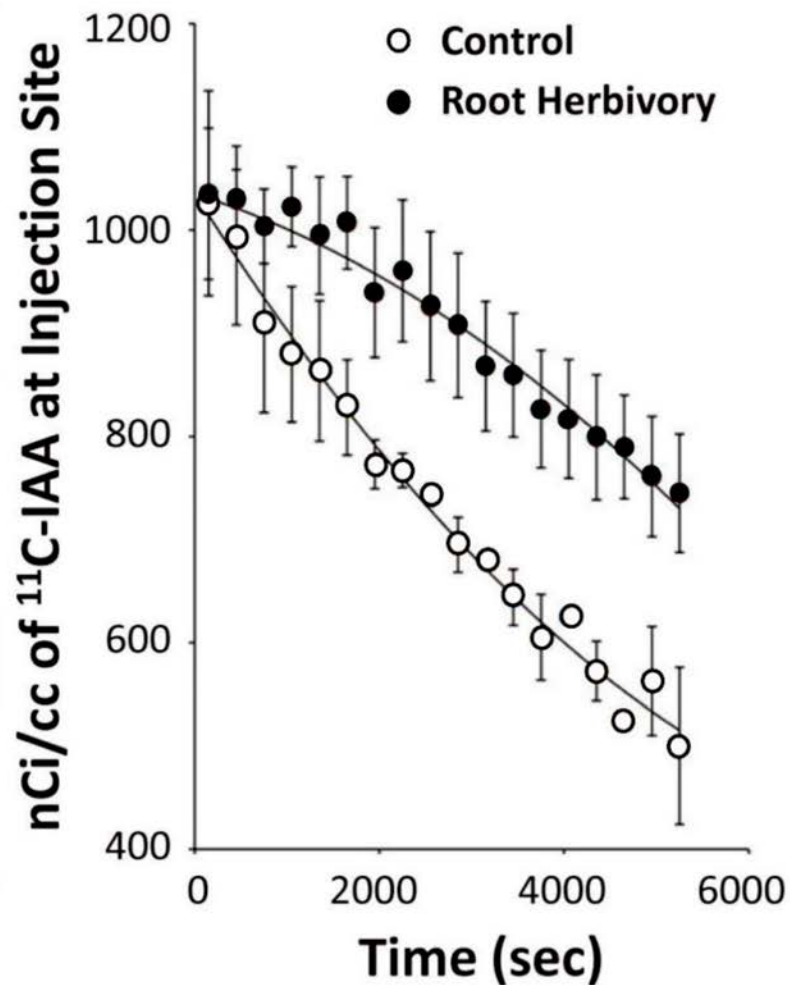


Figure 5. Panel a: Projection Positron Emission Tomography (PET) image showing ^{11}C IAA movement from the injection sites in the upper proximal zone of undamaged (control) nodal roots. Panel b: time-activity plots reflecting levels of decay corrected ^{11}C IAA activity at the injection site over time in seconds (N=4, control; N=5, root herbivory). Root herbivory decreased ^{11}C IAA transport 2.6-fold from an average speed of $17.0 \pm 5.7 \text{ mm hr}^{-1}$ as measured from time-of-arrival of the activity from across two distinct ROIs along an individual root.

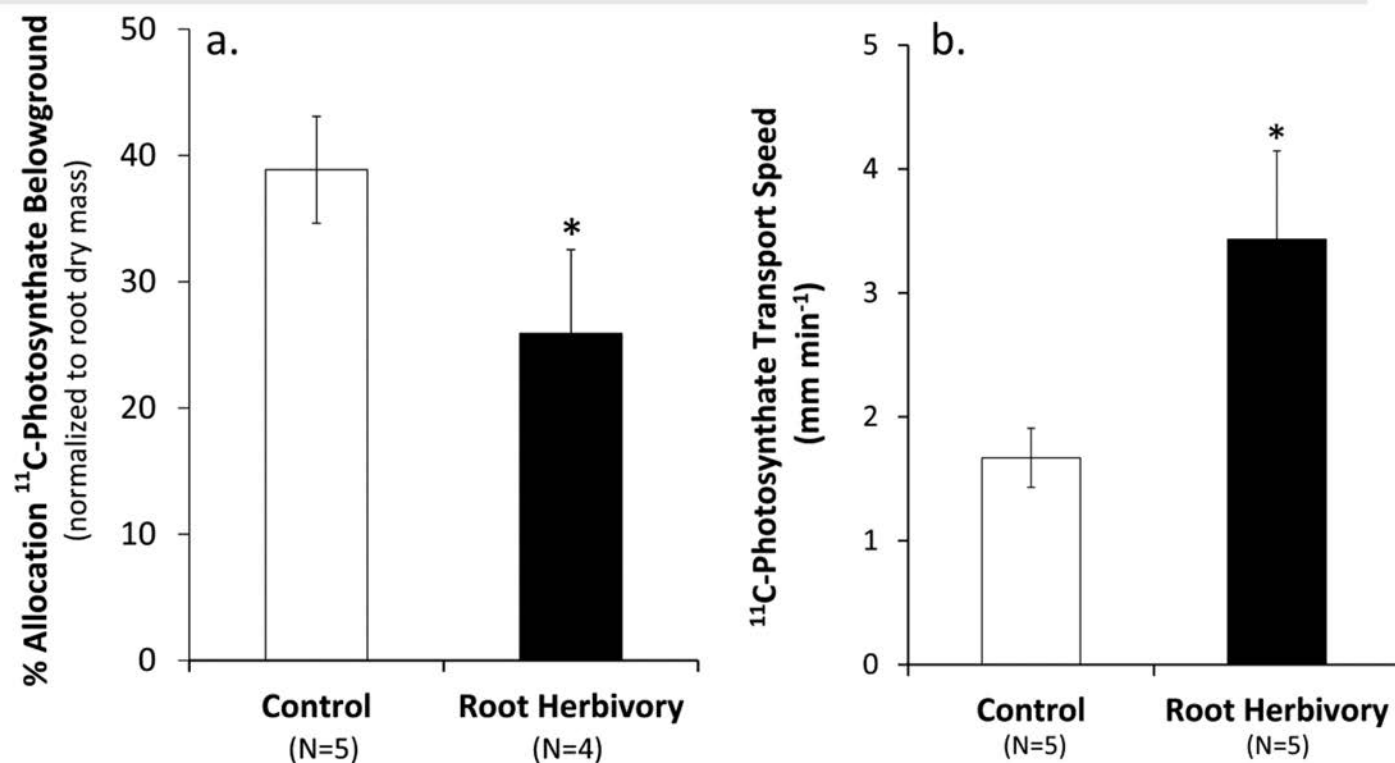


Figure 6. Panel a: allocation belowground of gross [^{11}C]photosynthates derived from fixed $^{11}\text{CO}_2$. Root herbivore treatment significantly decreased belowground allocation of these resources. Panel b: the speed of [^{11}C]photosynthate transport (mm min^{-1}) shown in panel b increased significantly with treatment. Where appropriate, statistical significance was denoted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

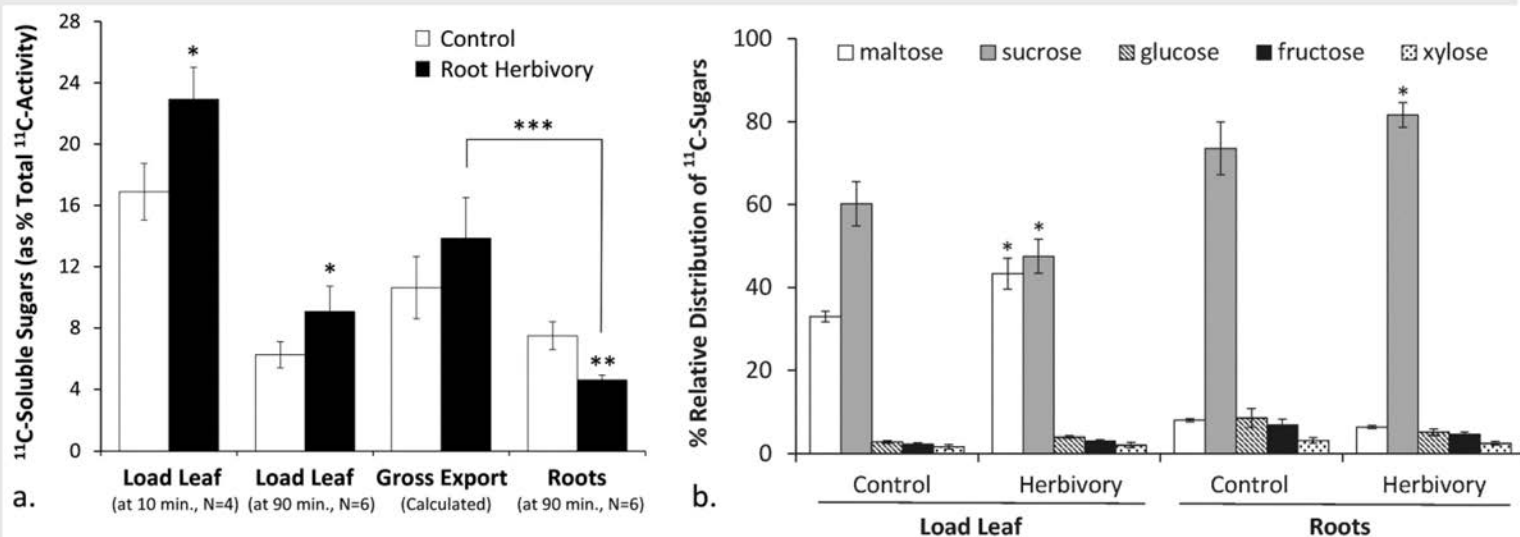


Figure 7. Panel a: levels of ^{11}C -labeled soluble sugars (presented as % total ^{11}C activity fixed as $^{11}\text{CO}_2$) were plotted for the following tissues: load leaf at 10 min. post-administration of $^{11}\text{CO}_2$; load leaf at 90 min. post-administration of $^{11}\text{CO}_2$; and roots at 90 min. post-administration of $^{11}\text{CO}_2$ to leaves. The gross export of ^{11}C -labeled soluble sugars was calculated from the difference in load leaf activities at 10 and 90 minutes. Root herbivore treatment manifested in a significant reduction in root allocation of ^{11}C -labeled soluble sugars. Panel b: relative distributions of ^{11}C -labeled soluble sugars are presented for the load leaf and roots at 90 min. post-administration of $^{11}\text{CO}_2$ and as a function of root herbivore treatment.

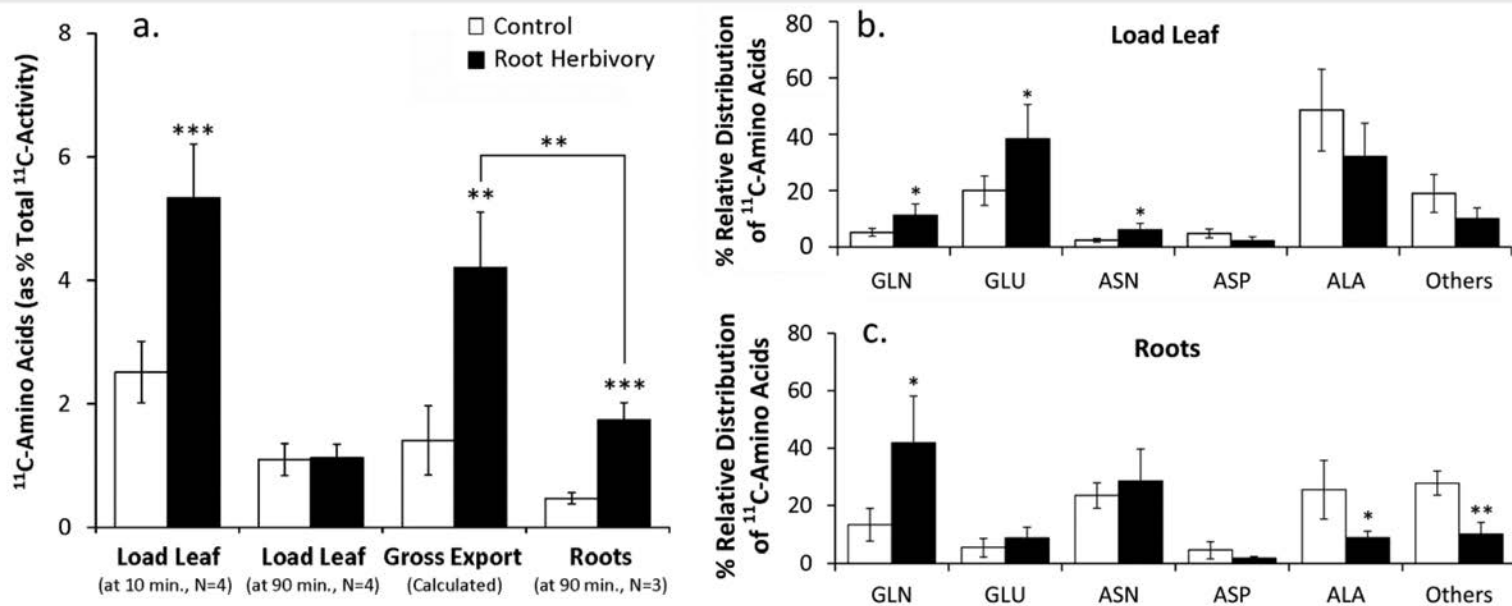


Figure 8. Panel a: levels of ^{11}C -labeled amino acids (presented as % total ^{11}C activity fixed as $^{11}\text{CO}_2$) were plotted for the following tissues: load leaf at 10 min. post-administration of $^{11}\text{CO}_2$; load leaf at 90 min post-administration of $^{11}\text{CO}_2$; and roots at 90 min. post-administration of $^{11}\text{CO}_2$ to leaves. The gross export of ^{11}C -labeled amino acids was calculated from the difference in load leaf activities at 10 and 90 minutes. Root herbivore treatment manifested in a significant increase in gross export of amino acids from source leaves, as well as in a significant increase in their allocation to roots. Relative distributions of ^{11}C -labeled amino acids are presented for the load leaf (Panel b) and roots (Panel c) at 90 min. post-administration of $^{11}\text{CO}_2$ and as a function of root herbivore treatment. Treatment increased source leaf levels of [^{11}C]glutamine, [^{11}C]glutamate and [^{11}C]asparagine. Treated roots showed significantly higher levels of [^{11}C]glutamine, as well as lower levels of [^{11}C]alanine and other less abundant radiolabeled amino acids that were summed together. Where appropriate, statistical significance was denoted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

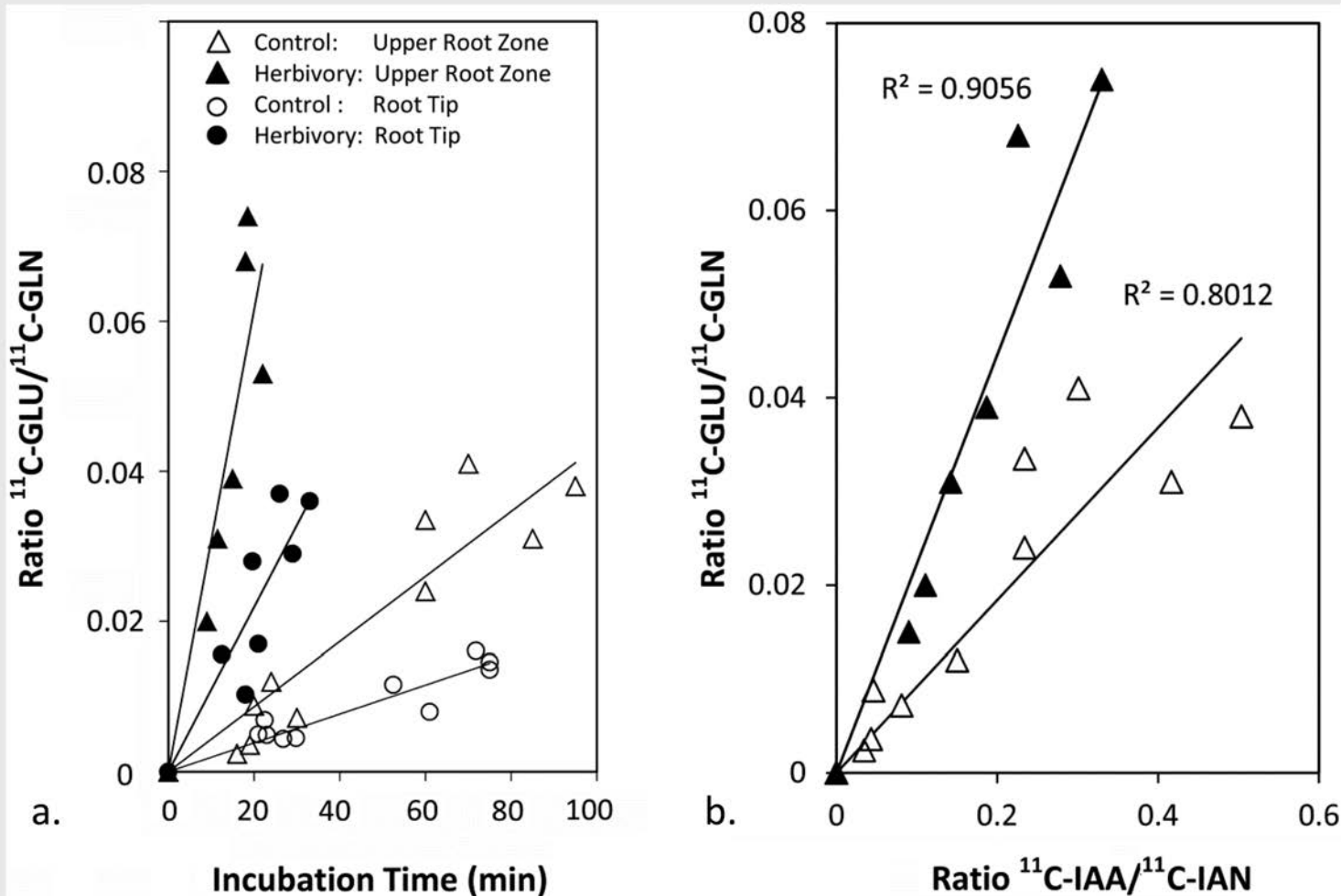


Figure 9. Panel a: [^{11}C]Glutamine metabolism, represented as the ratio of [^{11}C]GLU-to-[^{11}C]GLN, was plotted against tracer incubation for upper root tissues and root tips as a function of root herbivore treatment. Two key observations include: (i) GLN metabolism is higher in upper root tissues than in tips; (ii) root herbivore treatment increases GLN metabolism across all spatial zones of the root. Panel b. [^{11}C]GLN metabolism, represented as the ratio of [^{11}C]GLU-to-[^{11}C]GLN was plotted against [^{11}C]IAN metabolism, represented as the ratio of [^{11}C]IAA-to-[^{11}C]IAN, as a function of root herbivore treatment. Regression analysis reveals a strong correlation between the two biochemical processes as well as a significant positive herbivore treatment effect. Abbreviations: GLN, glutamine; IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile.

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